

Cholesterol crystal formation and growth in model bile solutions

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Abstract Cholesterol monohydrate crystal formation was studied in supersaturated model bile solutions, containing unlabeled cholesterol, sodium cholate and soybean phosphatidylcholine, and tracer amounts of [³H]cholesterol. Solutions were either seeded with cholesterol crystals to initiate growth, or not seeded to allow self-nucleation and subsequent crystal growth to occur. Crystal growth at 37°C was measured by two methods. First, radioactive cholesterol crystals were isolated by filtration, and the mass of cholesterol that had precipitated was calculated. In unseeded solutions, there was a long lag period before crystal growth was detected. This lag time was decreased by increases in the cholesterol concentration, temperature, and lipid concentration. In seeded solutions, crystal growth also was dependent on the cholesterol concentration, temperature, and lipid concentration. The second method used to measure crystal growth involved the Coulter Counter. At 37°C, reproducible results were not obtained using unseeded solutions due to blocking of the counter aperture with large crystals. In seeded solutions, crystal growth could be measured as an increase in total particle volume. However, comparison of growth rate estimates from the Coulter Counter with those obtained radiochemically revealed poor agreement between the two methods. It is probable that the Coulter Counter is inaccurate in measuring the volume of cholesterol monohydrate crystals due to their anisometric shape.—**Whiting, M.J., and J. McK. Watts.** Cholesterol crystal formation and growth in model bile solutions. *J. Lipid Res.* 1983. **24**: 861–868.

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Two of the stages in the development of cholesterol gallstone disease are the chemical stage, which involves the production of gallbladder bile that is supersaturated with cholesterol, and the physical stage, in which bile is nucleated and the growth of cholesterol monohydrate crystals begins (1). Further growth and aggregation ultimately lead to the formation of macroscopic cholesterol stones. In bile from gallbladders containing cholesterol stones, cholesterol crystals are detected in over 80% of cases (2).

Although the chemical stage is a prerequisite for the physical stage, the presence of supersaturated bile does not necessarily lead to the immediate formation of cho-

lesterol crystals. It is now well recognized that the gallbladders of subjects without gallstones may contain supersaturated bile without crystals, especially after fasting (3, 4). In addition, supersaturated bile samples can be incubated at 37°C for several days in the laboratory without the appearance of cholesterol crystals (5, 6). The reason for this metastability of apparently supersaturated bile is not clear, but it has been proposed that inhibitors of cholesterol crystal nucleation and growth may be present in human bile (1, 5, 6). The testing of this hypothesis requires an experimental system in which the formation of cholesterol crystals can be measured quantitatively. The aims of this work were to develop such a system using model bile solutions, and to compare a radiochemical method with the Coulter Counter for the measurement of crystal growth. The results of some of this work have been published in abstract form (7).

MATERIALS AND METHODS

Materials

The three lipids used to prepare the model bile solutions were cholesterol (melting point 146.5–147°C; Steraloids Inc., Wilton, NH), sodium cholate (98% purity; Sigma Chemical Co., St. Louis, MO), and soybean phosphatidylcholine (95% purity; Nattermann & Cie GmbH, Cologne, West Germany). The phosphatidylcholine contained a high proportion of unsaturated fatty acids, especially linoleic acid (8). The molecular weight calculated from the fatty acid composition was 793. Stock solutions of these lipids were prepared at the following concentrations, and stored at 4°C in the dark: cholesterol (50 mg/ml in chloroform); sodium cholate (200 mg/ml in methanol–water 85:15 (v/v)); phosphatidylcholine (500 mg/ml in chloroform–methanol 2:1 (v/v)). Radioactive [7(n)-³H]cholesterol, sp act 8 Ci/mmol, was obtained from Amersham Australia, Sydney, New South Wales, in toluene solution. Glass micro-fibre

filters (GF/C, retention efficiency 1.2 μm) were supplied by Whatman Inc., Kent, England.

Preparation of model bile solutions

Model bile solutions were prepared by coprecipitation of lipids in a manner similar to that described by Toor, Evans, and Cussler (9). Different volumes of each lipid solution were mixed together to achieve the desired mole ratio: sodium cholate–phosphatidylcholine 8:2, with cholesterol varying from 0.00 to 0.68. In addition, 2.5 μCi of radioactive cholesterol was added as tracer to each solution. Solvents were removed by heating solutions to 60–65°C under a stream of nitrogen. To remove final traces of solvent, the lipids were lyophilized under a vacuum of less than 0.5 torr for 4–6 hr. The dry solids (total weight 1.0 g) were stored in sealed vials at –15°C.

Micellar solutions of cholesterol were prepared by adding distilled water or electrolyte (0.15 M NaCl or KCl) to a dry coprecipitated sample to a volume of 10.0 ml (final concentration 10% by weight in solids). Samples were heated in a water bath to 55–60°C for 1 hr, and the resultant clear solutions were filtered through a 0.22- μm Millex-GS filter (Millipore Corp., Bedford, MA) into sterile vials sealed with a screw-cap. Cholesterol was completely dissolved by this procedure since the amount of radioactive cholesterol in filtered solutions was equivalent to the amount initially added. The clear supersaturated solutions were placed in an oven at 37°C and incubated for several days to allow the formation of cholesterol crystals. In some experiments, cholesterol monohydrate seed crystals (100 μl) were added to the solutions to initiate crystal growth at 1 hr after placing in the oven.

Preparation of cholesterol monohydrate seed crystals

Cholesterol monohydrate crystals were prepared as described by Igimi and Carey (10). Five g of cholesterol was dissolved in 400 ml of ethanol at 60°C and the solution was slowly cooled to room temperature. After several days, large flat cholesterol crystals were harvested by filtration and resuspended in 40 ml of distilled water. To reduce crystal size, the suspension was sonicated six times for 10 sec each at a power output of 50 watts (Branson Sonic Power Co., Danbury, CT). The suspension then was centrifuged at 1,000 g for 5 min and the supernatant was collected. The crystals that had sedimented were resuspended, and the sonication and centrifugation were repeated. The cloudy supernatants were pooled and stored undisturbed at 4°C for 4 days. During this time, a small amount of sediment formed and the supernatant was carefully decanted before being adjusted to 100 ml with distilled water. Sodium

azide (10 mg) was then added and the seed crystal suspension was stored at 4°C.

Chemical analysis by gas–liquid chromatography showed that the cholesterol concentration of the crystal suspension was 0.12 mg/ml. All crystals were less than 5 μm in diameter when examined by polarizing microscopy. This observation was confirmed with the Coulter Counter, which showed that the suspension contained 4.0×10^6 particles/ml in the size range 2 to 5 μm , with 68% of the counted particles being less than 2.5 μm in diameter. The detection limit of the counter under the conditions used was 2.0 μm so that any smaller particles were not measured.

Microscopic examination of model bile solutions

Samples of model bile (50 μl) were placed on warm microscope slides and immediately examined by bright-field and polarizing microscopy, using a Leitz SM-Lux microscope equipped with a measuring eyepiece.

Assay of cholesterol crystal growth

Radiochemical assay. At various incubation times, 400 μl of model bile solution, containing radioactive cholesterol in micellar solution and in crystalline form, were passed through a glass micro-fibre filter. The crystals trapped on the filter were washed three times with 4 ml of 0.9% (w/v) NaCl prior to scintillation counting in a Searle Mark III 6880 liquid scintillation counter at an efficiency for ^3H of 37%. The coefficient of variation after filtering and counting the same samples six times by this method was 2%. Since the specific activity of cholesterol in each model bile solution was known, the mass of crystals in the solution was calculated and expressed as mg of cholesterol per 10 ml. Growth rates then were measured as increases in the mass of cholesterol crystals per day.

Coulter Counter assay. Particle number and size distributions were measured on a calibrated Coulter Counter, model TA II with population accessory (Coulter Electronics Ltd., Hertfordshire, England), using a 70- μm aperture tube (particle diameter detection range 2 to 32 μm). To avoid errors due to coincident particle passage, samples from model bile solutions were diluted up to 200-fold with filtered (0.2 μm) 0.15 M NaCl in order to work in the linear response range of the counter. Replicate dilutions ($n = 6$) of the same sample gave a coefficient of variation of 2% in the number of particles counted. The total number of particles per ml of undiluted model bile was recorded, along with the size distribution of particles. Growth was measured as an increase in the total particle volume of crystals, based on particle diameters derived by the counter from particle volumes on the assumption that the particles were spheres.

RESULTS

Unless otherwise stated, all results presented below were obtained using model bile solutions that were prepared in 0.15 M NaCl at pH 8.2 and 10% (w/v) lipid concentration, with the sodium cholate–phosphatidylcholine mole ratio fixed at 8:2.

Crystal formation in unseeded solutions

In solutions that were incubated at 37°C without cholesterol monohydrate seed crystals, daily microscopic examination revealed the appearance of birefringent, parallelogram-shaped cholesterol crystals after several days. Some single crystals were observed, but usually crystals were clustered in large aggregates with spiral dislocation growth patterns and twinning also being observed (9). The size of the crystal plates varied from around $5 \times 5 \mu\text{m}$ to up to $300 \times 300 \mu\text{m}$ after 7 days incubation.

Radiochemical assay

There was a long lag time before cholesterol crystals were detected in unseeded model bile solutions using the radiochemical assay method (Fig. 1). This lag time represents the time interval in which micronuclei of cho-

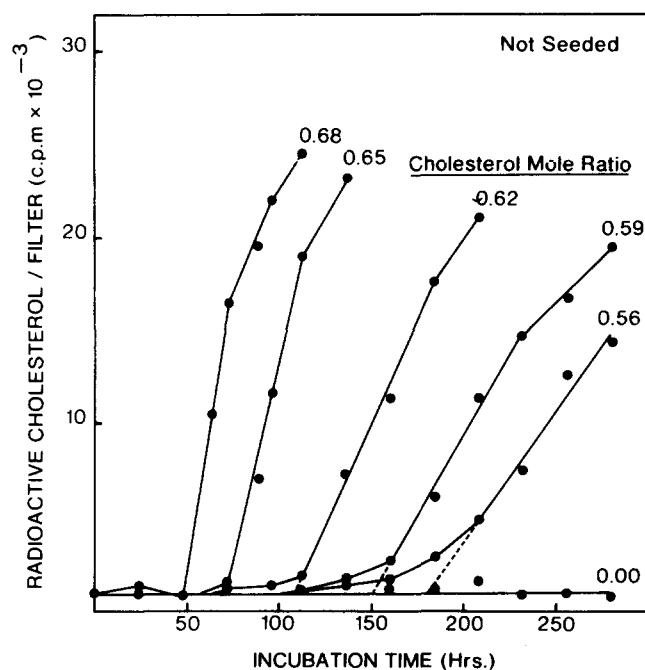


Fig. 1. Cholesterol crystal formation in unseeded model bile solutions as determined radiochemically. Solutions contained sodium cholate and phosphatidylcholine in the fixed mole ratio of 8:2, and different amounts of cholesterol (mole ratio 0.00 to 0.68). The lipids were dissolved in 0.15 M NaCl at a lipid concentration of 10% and pH 8.2, and solutions were incubated at 37°C. At various times, 400- μl samples of each solution were filtered and the amount of radioactive cholesterol crystals per filter was determined.

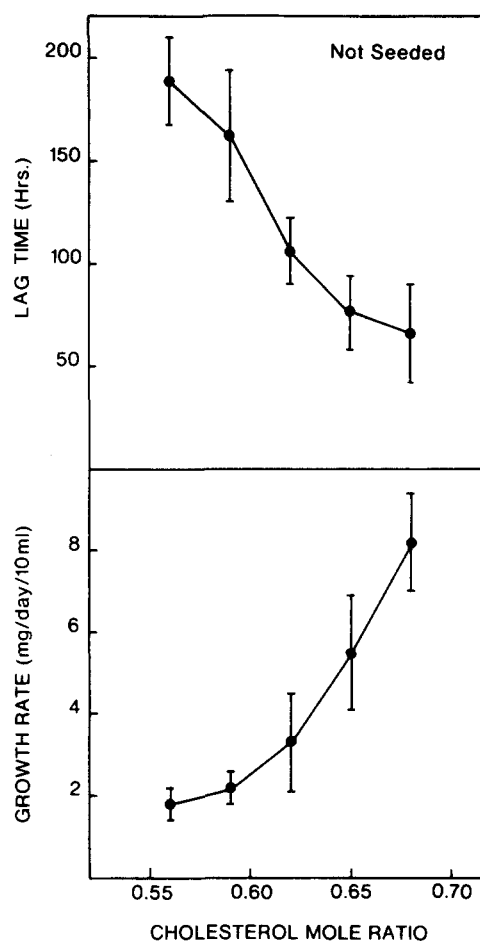


Fig. 2. Lag time and cholesterol crystal growth rate in unseeded solutions according to the mole ratio of cholesterol. Model bile solutions were prepared and incubated as described in the legend to Fig. 1, and the lag time prior to linear growth and initial linear growth rate of cholesterol crystals were determined at five different mole ratios of cholesterol. Results are expressed as the mean \pm SD of five experiments.

lesterol monohydrate have formed and grown to a detectable size. The lag time necessary to reach a linear crystal growth rate was related to the degree of cholesterol supersaturation, and in five experiments varied from a mean of 67 ± 24 (SD) hr at a cholesterol mole ratio of 0.68 to 189 ± 21 hr at a ratio of 0.56 (Fig. 2). The crystal growth rate was also related to the cholesterol mole ratio and increased 4-fold when the ratio changed from 0.56 to 0.68 (Fig. 2).

The lag time was affected by other variables encountered in the preparation of the model bile solutions. When water was used as electrolyte instead of NaCl, at a fixed cholesterol mole ratio of 0.62, the lag time to reach linear growth was increased to a value of 188 ± 19 hr compared to 106 ± 16 hr in NaCl ($n = 5$). However, solutions prepared in 0.15 M KCl were not different from those prepared in 0.15 M NaCl. This

result does not agree with the findings of Toor et al. (9), who found that no crystals appeared in KCl solutions after 15 days at a cholesterol concentration at which cholesterol crystals appeared after 1 day in NaCl. With water as the electrolyte, decreasing the incubation temperature from 37 to 22°C further increased the lag time from 188 ± 19 hr to 318 ± 5 hr ($n = 3$) at a cholesterol mole ratio of 0.62. Lag times at 37°C were less when the total lipid concentration was 20% (132, 144 hr, $n = 2$) compared to 5% (206, 216 hr, $n = 2$).

Coulter Counter assay

In unseeded solutions incubated at 37°C, difficulties were encountered in obtaining reproducible counting of particle numbers with the Coulter Counter, especially after incubation periods of a few days and at higher cholesterol concentrations. This was due to large crystals or crystal aggregates blocking the aperture (70 μm diameter) of the counter. Toor et al. (9) have reported using the Coulter Counter to study cholesterol crystal growth at 23°C in model bile solutions containing sodium taurocholate. When incubated at room temperature (21–23°C), the model bile solutions prepared in the present study formed a cholesterol precipitate composed of small (<10 μm) cholesterol crystals with a typical parallelogram shape, as well as long, thin, needle and hair-like crystals.

The particle number and size distribution in these solutions were determined without dilution, as described by Toor et al. (9). In agreement with these workers, particle size distribution curves were logarithmic normal and yielded a mean particle diameter that increased linearly with incubation time. However, when crystal suspensions were diluted up to 500-fold with saline, the mean particle diameter decreased markedly and the particle number was only slightly decreased. For example, in a solution containing cholesterol, sodium cholate, and phosphatidylcholine in the mole ratio of 0.66:8:2, respectively, the mean particle diameter after 3.8 days incubation at 22°C was 7.2 μm in undiluted solution. This value decreased to 6.2, 3.8, 3.2, and 2.6 μm at dilutions of 10, 100, 200, and 500-fold, respectively, while the particle number only decreased by half over this dilution range. These results are explained by coincident particle passage which occurs when visibly cloudy, undiluted crystal suspensions are used in the Coulter Counter. Under these conditions, several small particles may be counted as one large particle and any data obtained are inaccurate.

Crystal formation in seeded solutions

To reduce the long lag time observed prior to measurable crystal growth in studies with unseeded solutions, the experiments described in Figs. 1 and 2 were

repeated under identical conditions except that cholesterol monohydrate seed crystals (100 μl) were added to initiate crystal growth. Microscopic examination of seeded bile solutions showed that the small crystals added increased in size from less than $5 \times 5 \mu\text{m}$ to up to $50 \times 50 \mu\text{m}$ after 7 days. Compared to unseeded solutions, crystals were smaller and more dispersed, and twinning and aggregation were less common.

Radiochemical assay

At various incubation times after seeding model bile solutions containing various amounts of cholesterol, the amount of radioactive cholesterol crystals in suspension was determined (Fig. 3). As expected, the lag time prior to the measurement of a linear growth rate of cholesterol crystals was greatly reduced compared to unseeded solutions. In solutions containing cholesterol in the mole ratio range 0.56 to 0.68, the initial crystal growth was linear with incubation time for at least 24 hr, after which growth slowed. The initial growth rate was linearly related to the mole ratio of cholesterol over the range studied, and varied from 1.0 ± 0.3 mg/day per 10 ml of solution at a mole ratio of 0.56 to 6.5 ± 0.6 at a mole ratio of 0.68 (see Fig. 4). Inter-experimental variation for crystal growth rates was much less in seeded solutions than in unseeded solutions.

In addition to the cholesterol concentration, the incubation temperature, lipid concentration, and the

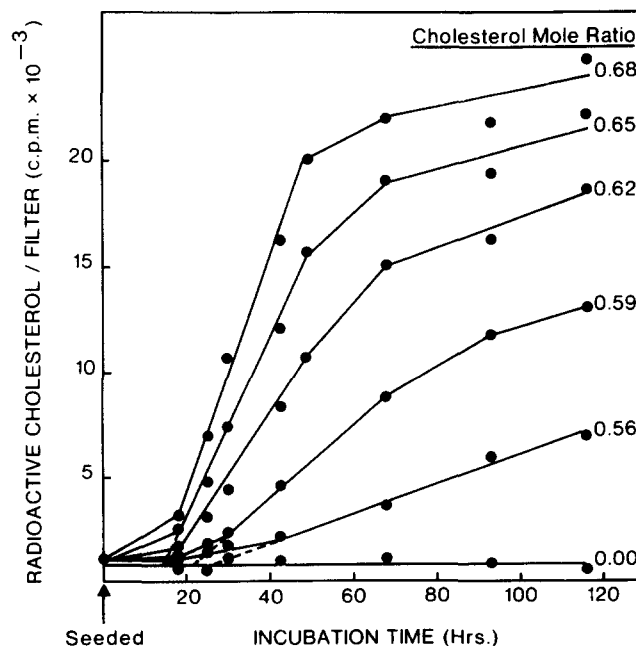


Fig. 3. Cholesterol crystal formation in seeded solutions as determined radiochemically. Model bile solutions were prepared and incubated exactly as described in the legend to Fig. 1, except that at zero incubation time, 100 μl of seed crystal suspension was added to the solutions to initiate growth.

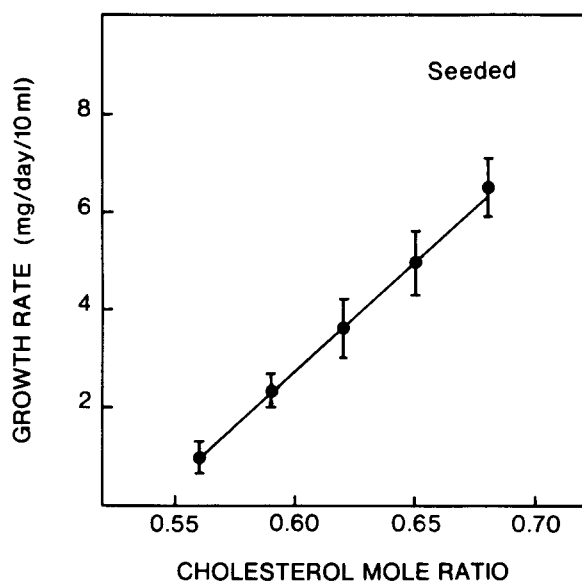


Fig. 4. Cholesterol crystal growth rate in seeded model bile solutions according to the mole ratio of cholesterol. Model bile solutions were prepared and incubated as described in the legend to Fig. 1, and the linear growth rate of cholesterol crystals was determined at five different mole ratios of cholesterol. Results are expressed as the mean \pm SD of five experiments.

number of seed crystals added to the model bile solution all were noted to affect the cholesterol crystal growth rate. In three separate experiments at a fixed cholesterol mole ratio of 0.62, the growth rate at 22°C was 2.0 ± 0.2 mg/day per 10 ml solution, which was less than half the growth rate at 37°C. Varying the lipid concentration of the solution from 5 to 20% increased the growth rate from 2.1 ± 0.3 to 5.4 ± 1.0 mg/day per 10 ml solution, while increasing the number of seed crystals 8-fold from 50 μ l to 400 μ l per 10 ml solution increased the growth rate by a factor of 2. Variables found not to affect the cholesterol crystal growth rate were the pH of the solution (range 7.5 to 9.5), and the electrolyte (water, 0.15 M NaCl, or KCl).

Coulter Counter assay

To complement the crystal growth rate measurements performed with the radiochemical assay, samples from the seeded model bile solutions described in Fig. 3 were diluted up to 200-fold with filtered saline, and the particle number and size distributions were determined. All samples were counted twice and the average percent variation between the readings for 20 samples was $2.8 \pm 2.1\%$. The particle number was linearly related to sample dilution and there was no change in the relative particle size distribution over the dilution range used.

As shown in Fig. 5, there was a large increase in the number of particles per ml of model bile solution de-

tected by the counter following seeding. The rate of increase was greater at higher mole ratios of cholesterol. After 40 hr incubation time, the particle number decreased in solutions of higher cholesterol concentration (mole ratio 0.62, 0.65, 0.68). This decrease can be explained by aggregation of cholesterol crystals, as particle size distributions showed an increased proportion of particles had larger diameters as the incubation proceeded (see Table 1).

As the Coulter Counter actually measures particle volume and calculates particle diameter distributions on the assumption that particles are spheres, growth is best defined as an increase in particle volume, and not diameter. The total particle volume was therefore calculated for each sample, and is shown in Fig. 6. After an initial lag, a linear increase in particle volume was observed at each mole ratio of cholesterol over a similar range of incubation times as observed when growth was measured radiochemically (see Fig. 3).

Comparison of assays of crystal growth in seeded solutions

Particle volumes measured by the Coulter Counter were converted to particle mass, taking the density of cholesterol monohydrate to be 1.045 g/ml (11). Initial

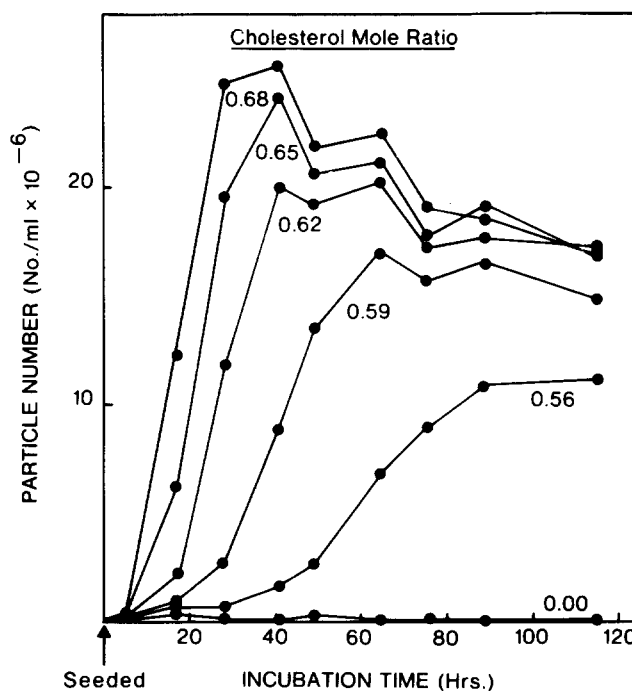


Fig. 5. Cholesterol crystal formation in seeded model bile solutions, determined as particle number with the Coulter Counter. Seeded model bile solutions were prepared and incubated as described in the legend to Fig. 3. At various incubation times, samples of model bile were diluted in filtered saline and the total particle number was determined at different mole ratios of cholesterol.

TABLE 1. Comparison of the size distributions of cholesterol crystals growing in seeded model bile solutions at three different cholesterol mole ratios

Incubation time	Mole Ratio Cholesterol	Size Distribution ^a Particle Diameter (μm)		
		2-4	4-8	8-32
<i>hr</i>				
0	0.56, 0.62, 0.68	96	4	0
17	0.56	95	5	0
	0.62	92	8	0
	0.68	85	14	1
28	0.56	96	4	0
	0.62	85	14	1
	0.68	54	34	12
41	0.56	95	5	0
	0.62	67	30	3
	0.68	47	35	18
65	0.56	90	9	1
	0.62	54	35	11
	0.68	41	37	22
89	0.56	72	26	2
	0.62	45	38	17
	0.68	38	39	23
115	0.56	65	32	3
	0.62	42	40	18
	0.68	41	37	22

^a Results are expressed as a percent of the total number of particles within the diameter range indicated, after various incubation times at 37°C. Each figure is the average of three experiments. Reproducibility between experiments was excellent with the average deviation from the figures shown being $1 \pm 1\%$. The three ranges of particle diameters (2-4, 4-8, 8-32 μm) corresponded to Coulter Counter channels 5-7, 8-10, and 11-16, respectively.

growth rates over a range of cholesterol mole ratios then could be expressed as mg cholesterol precipitated per day per 10 ml of model bile solution for both the radiochemical and Coulter Counter assay methods, allowing their direct comparison as shown in Fig. 7. As the cholesterol mole ratio increased, the cholesterol crystal growth rates obtained by the Coulter Counter were increasingly greater than those obtained by the radiochemical method.

DISCUSSION

Cholesterol crystal nucleation and growth from supersaturated gallbladder bile are two of the stages leading to gallstone formation (1, 5). There have been few studies that have attempted to measure these processes, although cholesterol monohydrate dissolution by bile acid solutions has been studied quantitatively (10). Recently, there has been renewed interest in cholesterol crystal formation, since bile from patients without gall-

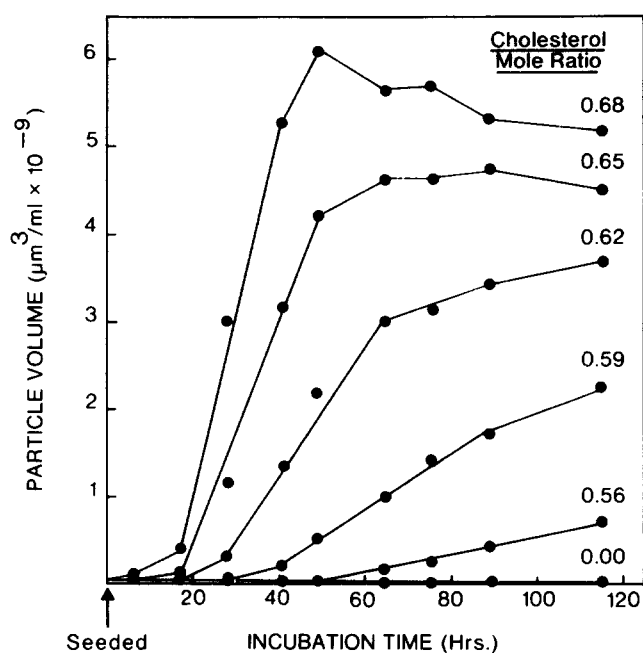


Fig. 6. Cholesterol crystal formation in seeded model bile solutions, determined as particle volume with the Coulter Counter. Seeded model bile solutions were prepared and incubated as described in the legend to Fig. 3. At various incubation times, samples of model bile solutions were diluted in filtered saline and the total particle volume was determined at different mole ratios of cholesterol.

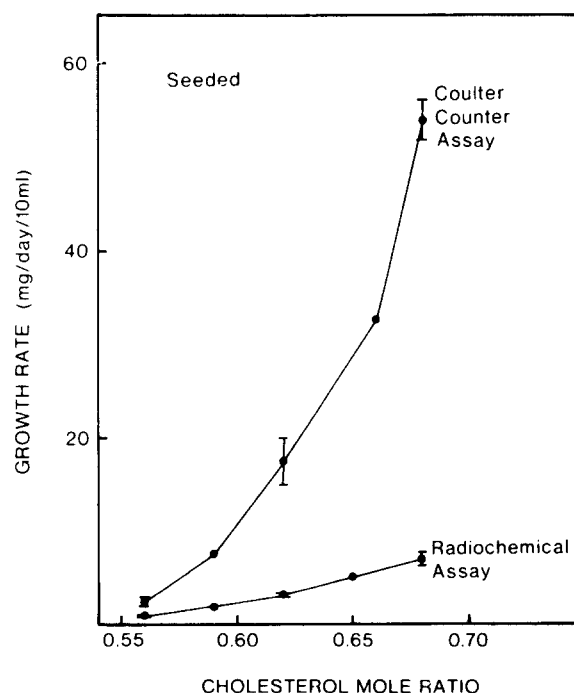


Fig. 7. Comparison of cholesterol crystal growth rates in model bile solutions containing different amounts of cholesterol, as estimated by radiochemical and Coulter Counter assay methods. The points at cholesterol mole ratios of 0.56, 0.62, and 0.68 are the mean \pm SD of three experiments.

stones can be apparently supersaturated and yet neither contain cholesterol crystals nor show a tendency to form cholesterol crystals when incubated in vitro (5, 6). This finding has led to the suggestion that there are inhibitors of cholesterol crystal nucleation and/or growth in gallbladder bile (1, 5, 6), but this possibility has not yet been explored.

The Coulter Counter is capable of particle size analysis and has been utilized to study calcium oxalate crystal growth (12) and cholesterol crystal growth (9, 13). In 1965, Higuchi and Saad (13) studied cholesterol crystal growth from saline solutions, and more recently, Toor et al. (9) grew cholesterol crystals from model bile solutions containing sodium taurocholate, egg phosphatidylcholine, and cholesterol. In our initial studies with the Coulter Counter, using unseeded solutions incubated at 22°C, we were able to reproduce the results of Toor et al. (9) using undiluted model bile solutions. However, these results appear to be inaccurate since particle diameters on which growth rate calculations were based were reduced dramatically when solutions were diluted to work within the linear range of the counter. At 37°C, the Coulter Counter could not be used to study cholesterol crystal growth in unseeded solutions under our conditions, as results were not reproducible owing to large crystals blocking the aperture of the counter.

When solutions were seeded with small cholesterol crystals to initiate growth at 37°C, reproducible results were obtained with the Coulter Counter. Solutions were diluted up to 200-fold in saline so that the particle number was linear with respect to sample dilution, and there was no change in relative particle size distributions. Under these conditions, growth could be measured as an increase in total particle number, but was better represented as an increase in total particle volume, since the electrical pulses measured by the Coulter Counter are proportional to particle volume (14). The increase in particle number may be due to the growth of very small seed crystals into the size range for detection by the Coulter Counter, or could represent promotion of crystal nucleation after seeding.

The radiochemical assay used in this work was developed as a simple and direct method for isolating cholesterol crystals and calculating their mass from the known specific activity of cholesterol in solution. This method was equally applicable to the measurement of crystal formation and growth from seeded and unseeded solutions, but gave no information on the size and number of crystals. Comparison of growth rates determined in the same seeded solutions by both the radiochemical and Coulter Counter assay methods revealed poor agreement over the range of cholesterol concentrations studied. At a cholesterol mole ratio of

0.68, the Coulter Counter growth rate of 51 mg/day per 10 ml solution was 7 times higher than that obtained by the radiochemical method. Detection limits of both assays were similar in that neither assay was capable of measuring particles smaller than 1 μm , and the pore size of the filters (1.2 μm) was close to the working limit (2.0 μm) of the Coulter Counter under the conditions used. It has been reported that the first particles precipitating from supersaturated model bile solutions are liquid crystals of cholesterol and phosphatidylcholine (15), and globular cholesterol microprecipitates of 700–2,000 Å diameter have also been observed prior to cholesterol monohydrate crystal formation (16). These particles would not have been detected in the present work.

In considering which of the two assay methods is likely to be accurate, the Coulter Counter method is suspect. This is because the response of the counter may not be proportional to particle volume when particles are highly anisometric (13), particularly if they are thin plates and crystal growth occurs along two rather than three dimensions, as is the case for cholesterol crystals (1). In any case, Coulter Counter growth rate estimates in solutions with a cholesterol mole ratio of 0.68 must be incorrect, since the amount of cholesterol that had precipitated from solution according to the counter exceeded the amount of cholesterol added in preparing the solution. Higuchi and Saad (13) also noted previously that volumes of cholesterol solids in suspension calculated from Coulter Counter data were 2 to 3 times greater than known amounts introduced for growth.

Using the radiochemical method, crystal growth rates were increased, and lag times decreased, by increases in cholesterol concentration, temperature, and the total lipid concentration of the solution. The greater than 2-fold stimulation of crystal growth observed at a lipid concentration of 20% compared to 5% appears paradoxical, since the degree of cholesterol saturation in model bile solutions decreases with increasing lipid concentration (4). However, if growth is diffusion-controlled (13), the greater number of micelles containing cholesterol in solutions of higher lipid concentrations would explain the increased growth rate of cholesterol crystals.

In conclusion, cholesterol monohydrate crystal formation can be studied using unseeded or seeded model bile solutions, but unseeded solutions require both nucleation and growth to take place before crystal formation will occur, while seeded solutions require only crystal growth. The mass of cholesterol crystals formed can be conveniently quantified radiochemically, and this experimental system should be useful in the study of potential inhibitors or activators of cholesterol crystal formation that may be present in human bile. However, Coulter Counter measurements of particle number and

volume must be interpreted cautiously, as it seems probable that the response of the counter is not proportional to the volume of these particles, owing to their highly anisometric shape. ■■

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